A Clinically Relevant in vivo Zebrafish Model of Human Multiple Myeloma (MM) to Study Preclinical Therapeutic Efficacy

Jianhong Lin¹,²,†, Weihong Zhang¹,²,³†, Jian-Jun Zhao¹, Ariel H. Kwart¹,², Chun Yang¹,²,⁴, Dongdong Ma⁵, Xiubao Ren³, Yu-Tzu Tai¹,², Kenneth C. Anderson¹,², Robert I. Handin⁵,‡, Nikhil C. Munshi¹,²,⁶‡

1. Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA
2. LeBow Institute for Myeloma Therapeutics and Jerome Lipper Center for Multiple Myeloma Research, Harvard Medical School, Dana-Farber Cancer Institute, Boston, MA, USA
3. Biotherapy Center, Tianjin Medical University Cancer Institute & Hospital, National Clinical Research Center of Cancer, Key Laboratory of Cancer Immunology and Biotherapy, Tianjin, P.R. China
4. Department of Clinical Laboratory, the 4th Hospital of Haerbin Medical University, Heilongjiang, China
5. Hematology Division, Department of Medicine, Brigham and Women’s Hospital, Boston, MA, USA
6. VA Boston Healthcare System, Boston, MA, USA

† These two authors contributed equally to this work

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‡ Address correspondence to:
Nikhil C. Munshi, Dana-Farber Cancer Institute, 450 Brookline Ave, Boston, MA 02215. Email: nikhil_munshi@dfci.harvard.edu.

Robert I. Handin, Hematology Division, Brigham and Women's Hospital, Karp Family Research Building, Rm 6210, One Blackfan Circle, Boston, MA 02115; e-mail: rhandin@partners.org.

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Key Points

- MM cell lines and primary MM cells can be engrafted and grown in vivo in Casper zebrafish larvae.
- Zebrafish MM in vivo xenograft model can be used as a pre-treatment drug sensitivity prediction platform for MM patients.
ABSTRACT

Patient-derived multiple myeloma (MM) cells are difficult to establish in culture or propagate in vivo in murine model. Here, we describe a zebrafish xenograft model that permits rapid, reliable growth of human MM cells injected into the perivitelline space of albino zebrafish (Casper) embryos, 48 hours post-fertilization. MM1S and MM1R MM cell lines and primary CD138+ MM cells, were stained with CM-Dil red fluorescent dye, suspended in Matrigel prior to their injection. The cells grow at the site of injection and disseminate throughout the developing embryos and larvae. Tumor size was quantified by fluorescent microscopy and cell fate was followed for 4 days. All of the cell line xenografts showed responses similar to those previously observed with in vitro assays. CD138+ plasma cell xenografts derived from newly-diagnosed MM patients also grew and were inhibited by the same drugs patients had responded to clinically. Using this technique, we can assess drug sensitivity or resistance with a small number of MM cells, in a short period. This raises the possibility that one might be able to assess drug sensitivity in real time with readily obtainable clinical samples.

Introduction

The gene expression profile and other genomic data have so far failed to predict individual responses to therapy1,2. Newer murine multiple myeloma (MM) models that recapitulate human disease to some extent have been developed but their ability to predict clinical responses have remained limited2-5. The limitations of murine tumor xenografts model includes the need for human fetal bone fragments to support the tumor growth, the low efficiency resulting in the generation of a limited number of tumor-bearing mice, and a two month lag period for tumor growth. These limitations highlight the need for a new model that allows efficient and rapid growth of human MM cells in an animal model applicable for pre-treatment drug response prediction and screening. Here, we describe a novel zebrafish model that supports the growth of both MM cell lines and primary MM cells and could provide an efficient, rapid and inexpensive platform for drug screening and studying the biology of MM.

Methods

Animal Care and Handling

The Casper strain of zebrafish (Danio rerio) were raised at 28.5°C in E3 buffer with cycles of 14/10 hours light/darkness cycles and fed in accordance with guidelines established by the Committee on the Ethics of Animal Experiments of Children’s Hospital Boston (No. 09-09-1540R).

MM cell lines and Patient cell Microinjection
All MM cell lines utilized and their culture conditions have been previously described\textsuperscript{6}. Primary plasma cells from MM patients were obtained after informed consent and isolated as described previously\textsuperscript{7}. The CM-DIL labeled MM cell lines and primary CD138+ MM cells were washed in PBS, re-suspended in Matrigel at 10,000 cells/µl. Thirty-six hour post fertilization (hpf) Casper embryos were dechorionated with 1 mg/ml of pronase for 5-10 minutes. Forty-eight to ninety-six hpf embryos were anesthetized by placement in 0.04 mg/ml Ethyl 3-aminobenzoate methanesulfonate tricaine. The MM cells (100 cells in 10nl, 5-20 nl injection volume/embryo) were injected into the perivitelline space with a pneumatic Pico-pump injector using glass micro-injection needles.

**Drug Treatment and Efficiency Evaluation**

We evaluated the tumor xenografts by fluorescence microscopy 24 hours post-injection (hpi). Those embryos with red fluorescence at the injection site were moved to 24-well uncoated plates (1 embryo/well) and freshly prepared E3 medium containing drugs or solvent was added to each well according to the experimental group. Stock solutions of the drugs to be tested were prepared, as previously described\textsuperscript{7-10} and then diluted at least at 1:1000 with E3 medium before adding to embryos in the 24-well plates. Fresh drug containing E3 medium was replaced daily. We evaluated tumor growth and fish survival at 24, 48 and 72 hours post treatment (hpt) by fluorescence microscopy. We measured the area and density of fluorescence on photomicrographs with ImageJ software and estimated tumor xenografts volume in control and drug treated animals.

**Results and Discussion**

**Casper zebrafish in vivo MM xenografts model**

We utilized transparent Casper zebrafish early stage embryos (48 hpf) as they allow \textit{in vivo} observation of growth changes in fluorescent-labeled myeloma cell xenografts (Figure 1A)\textsuperscript{11} and are immune deficient\textsuperscript{12,13} allowing the growth of human cells. We first utilized MM1S and MM1R MM cell lines to demonstrate MM cell growth in zebrafish embryos and determine whether addition of anti-MM drugs to embryo water would inhibit cell growth. As seen in Figure 1B, CM-Dil-labeled MM cells injected in perivitelline area of Casper zebrafish could be observed by fluorescence microscopy 24 hpi and over 80% of larvae retained injected MM cells for 9 days, providing an adequate window for drug screening. These xenografts were confirmed by immunofluorescence microscopy (Figure 1C).
We next examined the growth of MM1s cell line, huIL-6-dependent INA-6 MM cell line and purified CD138+ primary human MM cells in Casper zebrafish and observed their growth up to 96 hpi without addition of huIL-6 or BMSC (Figure 1D-E). Interestingly, the growth of MM cell lines and primary MM cells suggests that zebrafish embryo perivitelline space recapitulates the growth support provided by human BM microenvironment.

**Preclinical drug sensitivity studies in the zebrafish model**

We next evaluated whether this model could be utilized to assess efficacy of known anti-MM agent. We first evaluated the effect of dexamethasone on both dexamethasone-sensitive (MM1S) and dexamethasone-resistant (MM1R) MM cell lines. We established 100nM as the lowest concentration that inhibited the growth of MM1S cells. Up to 5µM dexamethasone did not affect survival of Casper zebrafish larvae for up to 3 days. We treated 11 MM1S and 11 MM1R xenograft in Casper zebrafish embryos with 100nM dexamethasone and the change in xenograft volume was estimated every 24 hours (Figure 2A-B). A 50% decrease in gross tumor volume from the size just prior to drug addiction was used as a “response” and an indicator of drug sensitivity. The data is then presented as number of fish responding out of the whole group at 24, 48 and 72 hpt. According to this method of analysis, the response of MM1S cells to dexamethasone was over 85%, while the response of MM1R to dexamethasone was 15%. This limited response was similar to that observed in the control group and consistent with previously published *in vitro* and *in vivo* studies. It just took 4 days from tumor inoculation to obtain this data which demonstrates the potential of this model to rapidly predict the sensitivity of MM cells to added drug.

To further evaluate the utility of the xenograft model for drug screening, we chose 2 first line anti-MM drugs, bortezomib (10nM) and lenalidomide (2µM), and 4 novel agents, AZD6244 (400nM), 17-AAG (800nM), AS703026 (800nM) and rapamycin (40nM), which have been tested in MM. We used 4 MM cell lines, MM1S, MM1R, OPM1 and RPMI8226, for this evaluation. MM1S is sensitive to bortezomib, lenalidomide and dexamethasone (>60% larvae showing a response); and relatively sensitive to all other 4 agents (30% to 60% of tested fish had a response) (Figure 2C). Relative sensitivity and resistance of other cell lines to the standard agents as well as investigational agents were shown in Figure 2D.

Next, we assessed the effect of these novel agents on primary MM cells from 2 newly diagnosed and 4 relapsed/refractory MM patients. Efficiency of engraftment of primary cells is similar to cell lines, which is about 80%. We treated embryos engrafted with patient cells with these agents and observed that both newly diagnosed patients responded to bortezomib and lenalidomide with >50% reduction at 72 hpt (Figure 2 E-F and data not shown). We observed that cells from those patients with resistance to
bortezomib and/or lenalidomide were also resistant to the same agents in our zebrafish model, validating the reliability of our system and confirming that primary MM xenografts can be used to predict patient responses to chemotherapy in vivo.

We here describe a zebrafish embryo xenograft model that supports the growth of MM cell lines and primary patient cells and can be used to predict drug sensitivity and potentially utilized for pre-clinical drug screening. The advantages of the system are ability to use patient cells, requirement for small number of MM cells, less variability between animals, ability to perform medium throughput in vivo drug screening using primary MM patient cells and short latency that may permit quick screening in real time. The limitations of this model include the smaller number of embryos that could be injected at single sitting, growth within a unique microenvironment, growth at a lower temperature required by fish and undervaluation of the influence on drug resistance induced by bone marrow microenvironment. The variability between different fish can be observed but can be compensated by injecting large number of larvae. The ability of zebrafish to support MM cell growth may provide insight into unique microenvironment in the fish that supports MM cell growth, even though it may not have all the characteristics of the actual bone marrow microenvironment. This easy, quick and economical model overcomes many of the limitations of murine xenograft models and should be further evaluated as a tool to screen and predict drug sensitivity in MM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Authorship contributions
J.L. and N.C.M. designed experiments, analyzed data, and wrote the paper; J.L., W.Z., J.J.Z., A.H.K, C.Y., and D.M. performed experiments and analyzed data; Y.T.T, and K.C.A. provided clinical samples. R.I.H. critically revised the paper.

References

Figure Legends

**Figure 1. Casper zebrafish supports MM cell growth.** (A) Schematic chart depicted the procedures to establish zebrafish MM xenograft model (hpf: hours post fertilization, hpi: hours post injection, hpt: hours post treatment). (B) Fluorescence microscopy images of the CM-Dil stained MM cells injected into perivitelline area of Casper zebrafish larvae. Upper: the bright field of whole Casper zebrafish 24 hours after injection with human MM cells. Middle: RFP channel of whole Casper zebrafish; the red fluorescence color under microscope is the CM-Dil stained MM cells. Lower: merged image from bright field and RFP filed. All cells are located in perivitelline area after mix with matrigel. (C) The whole mount IHC staining. Upper: the bright field of whole zebrafish 24 hpi with human multiple MM cells. Middle: the RFP field shows red fluorescence from CM-Dil, stained MM cells. Lower: the GFP field showing the EGFP-labeled anti-human lambda light chain antibody co-localizing with the CM-Dil labeled MM1S cells. (D) Typical fluorescence microscopy images of the CM-Dil stained MM cells xenograft growth of MM1S, INA-6, and primary patient MM cells without drug treatment in perivitelline area of Casper zebrafish larvae at 24, 48 and 72 hpi. (E) Tumor growth curves of xenografts with these 3 cell types in Casper zebrafish larvae (N=30 for each group).

**Figure 2. Response to standard and novel drugs in Zebrafish model.** (A) Fluorescent microscopy images of the CM-Dil stained MM1S and MM1R xenografts at 0, 24, 48 and 72 hpi. Tumor growth curves of MM1S and MM1R cells xenografts are showed in (B), which indicate MM1S xenografts are responsive and MM1R resistant to dexamethasone treatment. (C) % of MM1S xenograft without response in zebrafish after treatment with bortezomib (n=22), lenalidomide (n=22), dexamethasone (n=22), AZD6244 (n=18), 17-AAG (n=22), rapamycin (n=18) and AS703026 (n=18) and DMSO (n=20), respectively. Error bars indicate standard errors. (D) Summary of % of MM1S, MM1R, OPM1 and RPMI8226 larvae without response with various indicated novel agents. (E) Individual fluorescence microscopy images of CD138+ MM cell from a newly-diagnosed MM patient xenografts in zebrafish before and after treatment with bortezomib (n=8), lenalidomide (n=8), dexamethasone (n=8) and DMSO (n=8), at 0 hpt, 24 hpt, 48 hpt and 72 hpt, respectively. (F) % of primary patient MM cell xenografts without response to lenalidomide, bortezomib, and dexamethasone.
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